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# The evolutionarily conserved Krüppel-associated box domain defines a subfamily of eukaryotic multifingered proteins

(DNA-binding proteins/sequence conservation/cell differentiation)

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**ABSTRACT** **We have previously shown that the human genome includes hundreds of genes coding for putative factors related to the Krüppel zinc-finger protein, which regulates *Drosophila* segmentation. We report herein that about one-third of these genes code for proteins that share a very conserved region of about 75 amino acids in their N-terminal nonfinger portion. Homologous regions are found in a number of previously described finger proteins, including mouse Zfp-1 and *Xenopus* Xfin. We named this region the Krüppel-associated box (KRAB). This domain has the potential to form two amphipathic α-helices. Southern blot analysis of "zoo" blots suggests that the Krüppel-associated box is highly conserved during evolution. Northern blot analysis shows that these genes are expressed in most adult tissues and are down-regulated during *in vitro* terminal differentiation of human myeloid cells.**

Many DNA-binding proteins, involved in the regulation of gene transcription, are built around common structural motifs that are quite well conserved throughout evolution, even among highly divergent species.

Only a few such motifs have been found to mediate the characteristic DNA-binding properties of these proteins. The best known is the helix-turn-helix domain first discovered in prokaryotic repressors (1) and later in the homeobox regions of various eukaryotic proteins (2). A second DNA-binding motif is the zinc-finger repeat first found in *Xenopus* transcription factor TFIIIA (3) and subsequently found in a large number of eukaryotic regulatory proteins (4–8). Other DNA-binding domains present in dimeric proteins are the amphipathic helix-loop-helix (9) and a series of basic residues adjacent to a leucine zipper (10, 11); moreover, other types of DNA-binding motifs continue to be described (12–14).

The other motifs that are necessarily coupled to these DNA-binding domains to give the transcriptional factors their full function are less well characterized. Nevertheless, the few recognized to date seem to appear only in conjunction with a given DNA-binding domain, an observation that led to subdividing the original classification. For example, a number of homeobox proteins contain, next to their DNA-binding domain, other conserved modules such as the paired box (15) or the POU domain (16, 17). Among the large family of zinc-finger proteins, only one such associated motif (finger-associated box; FAX) of unknown molecular function has been found (18) in a number of *Xenopus* finger proteins that are potentially involved in early embryogenesis.

Herein, we report on the characterization* of a motif present at the N terminus of about one-third of the 300 zinc-finger proteins of the Krüppel type encoded in the human genome (19). This motif, which is in fact present in a number of finger proteins (20–23), is highly conserved in evolution and appears always associated with finger repeats.

## MATERIALS AND METHODS

**cDNA Library Screening.** A human λgt10 cDNA library from undifferentiated NT2D1 cells (24) was screened with a 546-base-pair *Eco*RI–*Eco*RI fragment corresponding to the 5′ nonfinger portion of the coding region of HPF4 (where HPF is human placental finger) (19). The DNA probes were labeled with a multiprimed labeling kit (Boehringer Mannheim) to a specific activity of $5 \times 10^8$ cpm/μg. Hybridizations were carried out at 65°C in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/5× Denhart's solution (1× Denhart's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.1% SDS and denatured salmon sperm DNA (100 μg/ml). Filters were washed under low-stringency conditions in 2× SSC/0.1% SDS at 50°C. Autoradiography was performed at −70°C for 48 hr with intensifying screens.

**DNA Sequencing and Sequence Analysis.** DNAs from positive clones were subcloned into M13mp18/19 or pGEM-3Zf(+/−) vectors (Promega). They were sequenced on both strands by using the dideoxynucleotide chain-termination method (Sequenase; United States Biochemical) on single-stranded or doubled-stranded DNA and universal or internal primers. Nucleotide and amino acid sequence analyses were performed using the Sequence Analysis Software Package (version 5) of the University of Wisconsin Genetics Computer Group.

**Northern and Southern Blot Analyses.** *Eco*RI-digested phage DNA fragments (1 μg) or 10 μg of *Eco*RI-digested genomic DNA were electrophoresed on a 0.8% agarose gel, transferred to a nylon filter (Amersham), and hybridized with the HPF4 Krüppel-associated box (KRAB) probe as described above. "Zoo" blots were also hybridized with a 1097-base-pair *Eco*RI–*Eco*RI fragment coding for fingers 3 to 14 of HPF4 (19); Southern blots of positive cloned cDNAs were also probed with the H/C link oligonucleotide as described (19). Total RNA was extracted from various adult human tissues and cell lines by the guanidinium isothiocyanate method (25). Samples (10 μg) of total RNA were electrophoresed on 1% agarose/formaldehyde gels and transferred to nylon filters (Amersham). The blots were hybridized with hexanucleotide-primed complete HPF4, HTF10, and HTF34 cDNAs (where HTF is human teratocarcinoma finger) as described for library screening. Filters were washed at 60°C in 0.1× SSC/0.1% SDS. 28S rDNA oligonucleotide (Clontech) and oligo(dT) (Eurogentec, Liège, Belgium)

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Abbreviations: KRAB, Krüppel-associated box; PMA, phorbol 12-myristate 13-acetate; HPF, human placental finger; HTF, human teratocarcinoma finger.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M61866–M61873).

probes were used to normalize the amount of RNA loaded. Autoradiography was performed at −70°C with intensifying screens. Exposure time was 6–12 hr for RNA blots and 1 week for genomic DNA blots.

**Cell Cultures.** THP-1 (acute promyelocytic leukemia) (26), HeLa (cervical carcinoma), and NT2D1 (embryonal carcinoma) (27) cells were cultured as described. THP-1 cells at 1 × $10^6$ cells per ml were induced to differentiate along the macrophage pathway by addition of 50 nM phorbol 12-myristate 13-acetate (PMA) to the culture medium [RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum].

## RESULTS AND DISCUSSION

Sequence analysis of nine human placental cDNAs encoding Krüppel-related finger proteins (19) revealed in two of them (HPF4 and HPF9) marked similarity (86% at the nucleotide level) in their 5′ nonfinger regions. We named these highly homologous regions KRAB. This unexpected homology led us to use the 5′ nonfinger portion of the coding region of HPF4 (spanning the putative KRAB domain) as a probe to screen a cDNA library derived from undifferentiated NT2D1 cells under low-stringency conditions (24). A total of 6 × $10^5$ recombinant phages was screened. A large number of these, about 0.03%, gave positive signals. Thirteen probe-positive recombinant phages were isolated and 11 of them were found to be distinct, as judged by their restriction patterns and by Southern blot analysis with either the HPF4 KRAB probe or with an oligonucleotide probe corresponding to the TGEKPY/F amino acid stretch (termed the H/C link) that connects successive finger loops in the *Drosophila* Krüppel finger protein (4) (Fig. 1). Moreover, as all positive clones detected by means of the HPF4 KRAB probe also yield positive signals with the H/C link probe, it may be assumed

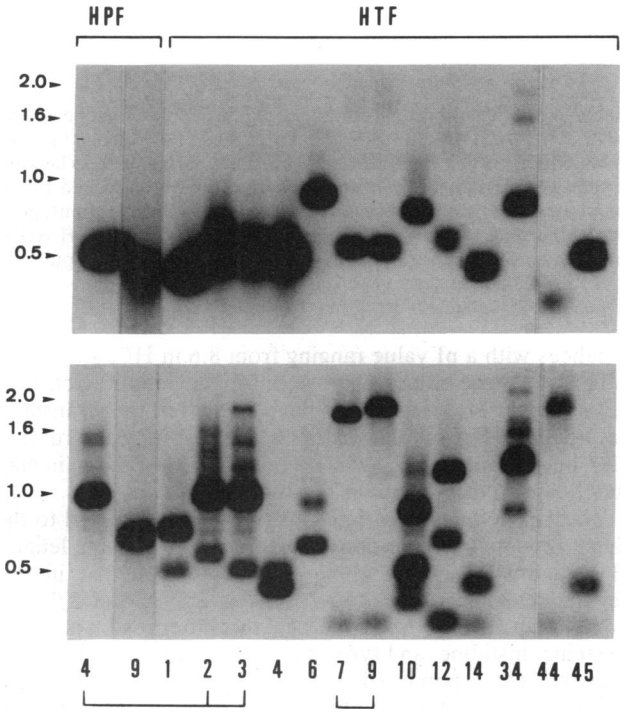


FIG. 1. Association of the KRAB domain with finger repeats. *Eco*RI digests of HPF4 KRAB-positive cloned cDNAs were analyzed on a Southern blot using either the HPF4 KRAB domain (*Upper*) or a Krüppel H/C link oligonucleotide (*Lower*) as probes. Previously isolated cDNAs HPF4 and HPF9 are indicated by lane labels. Brackets indicate cloned cDNAs with similar hybridization patterns. Molecular sizes in kilobase (kb) are shown to the left.

that the KRAB region is always associated with finger repeats. Positive clones obtained in this screening were therefore designated as HTF cDNAs.

To estimate the number of KRAB finger genes expressed in NT2D1 cells, we have (*i*) compared the total abundance of H/C-link-positive clones (1/1 × $10^3$) and KRAB-positive clones (0.3/1 × $10^3$) in the NT2D1 library, and (*ii*) estimated the number of distinct H/C-link-positive clones and KRAB-positive clones by screening the NT2D1 library under high-stringency conditions with various HPF or HTF cDNA probes so as to detect only identical or overlapping clones (i.e., HTF2 and HPF9 represent, respectively, 0.8% and 0.4% of the H/C-link-positive clones and 3.3% and 2.2% of the KRAB-positive clones). As our restriction pattern and sequence analyses exclude the possibility that a few highly expressed finger genes account for the majority of the KRAB-positive clones (11 out of 13 randomly picked HTF cDNA clones have distinct restriction patterns) or H/C-link-positive clones (19), our results strongly suggest that about one-third of the finger proteins detectable with a H/C link probe (4) contains domains whose sequence is homologous to that of the HPF4 KRAB region. A similar ratio was also obtained when analyzing the N-terminal sequences of the full-length placental cDNAs that we had previously isolated by hybridization with the Krüppel H/C link probe (19).

Six distinct HTF cDNAs ranging in size from 1.9 kb (HTF1) to 3.9 kb (HTF10) were chosen for further study. Sequence analysis showed that they all encode members of the Krüppel zinc-finger family. Comparison of equivalent regions (KRAB domain, finger region, and 3′ untranslated region) of these HTF cDNA clones clearly indicated that they were distinct from each other as well as from the characterized human finger proteins, with the exception of HTF10, which was identical to our previously identified Krüppel-related cDNA clone HPF7 (19). Fig. 2 shows the predicted amino acid sequences of each N-terminal nonfinger region and the first finger element. A search for homologies through the National Biomedical Research Foundation protein data bank revealed a striking homology with the N-terminal part of a number of finger proteins. These include the human *KOX1* gene expressed in human T cells and various nonhematopoietic cell lines (20): *ZNF7* and *ZNF8*, two human genes that are expressed in many cell lines and whose expression is modulated during *in vitro*-induced terminal differentiation of myeloid cells (21); *Zfp-1*, a ubiquitously expressed gene that may be implicated in early mouse embryogenesis (22); and *Xfin*, a gene that may be involved in the control of early embryonic development in *Xenopus* (23). Comparison of these 13 related sequences shows that, as for the finger-associated box domains (18), homology is strongest in the region closest to the N terminus. Starting at position 12 of HPF4 (Fig. 2*B*), 26 out of 75 amino acids are highly conserved.

By allowing for conservative substitutions, the degree of sequence similarity was increased to between 20% and 91%. Within this region of sequence similarity, two subregions with 46% to 92% (region A, Fig. 2*B*) and 14% to 94% (region B, Fig. 2*B*) amino acid identity could be distinguished due to either their absence from individual protein sequences [i.e., HTF6 lacks element A and HTF1 and HTF12 lack element B (Fig. 2*B*)] or their separation by variable spacer segments [i.e., spacer between elements A and B in HPF9 (Fig. 2*B*)]. Elements A–B are referred to as the KRAB domain. This organization of the KRAB domain is also supported by the observation that H-plk, a recently described HPF9-related transcript down-regulated in choriocarcinoma (28), contains a 5′ noncoding exon 89% identical to the A and B elements of the HPF4 KRAB domain with exon–intron splice sites corresponding exactly to the limit of the KRAB A element. Furthermore, in HTF34, a 204-base-pair fragment homologous to an *Alu* element with multiple stop

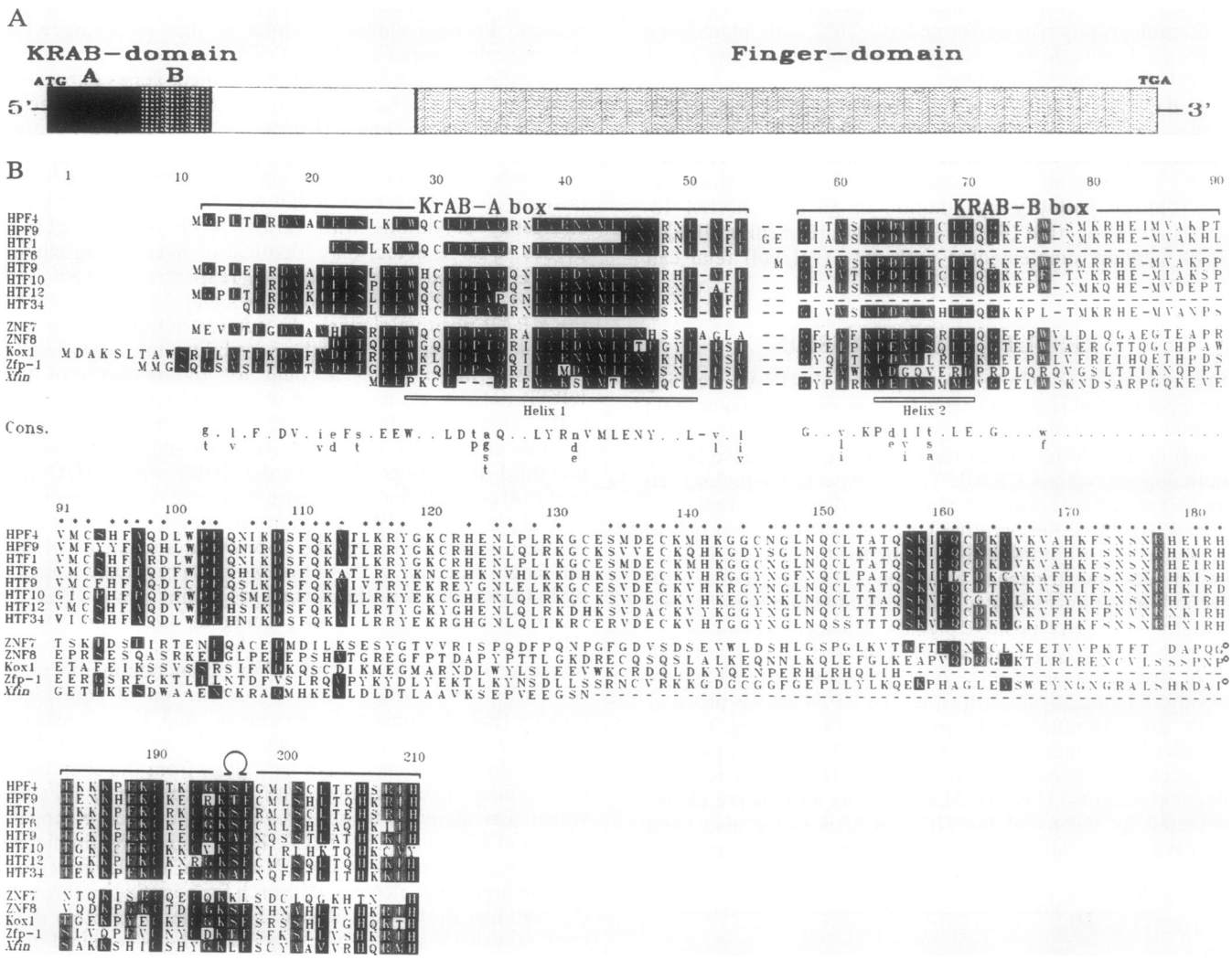


FIG. 2. Schematic representation of the HPF4 protein (*A*) and amino acid sequence of the N-terminal nonfinger region and first finger element (*B*) of eight KRAB-finger proteins. The sequences are compared with those of ZNF7, ZNF8, Kox1, Zfp-1, and Xfin. Most of the cloned cDNAs contain one open reading frame with the first methionine located in the A element; HPF9, HTF1, HTF10, and HTF34 are partial cDNAs lacking 5'-terminal sequences. The predicted protein sequence of Kox1 extends beyond the methionine in position 1, which is not preceded by an in-frame terminator codon. Sequences were aligned to HPF4. Black background indicates fully conserved residues (>75% conservation); gray indicates conservative amino acid residue substitutions (>75% conservation) according to the scheme (P,A,G,S,T), (Q,N,E,D), (H,K,R), (L,I,V,M), and (F,Y,W). KRAB boxes A and B are overlined and a consensus sequence (Cons.) for all 14 proteins is presented. The putative $\alpha$-helical regions are represented below the sequence. In the region connecting the KRAB and finger domains, identical or similar residues (>75% conservation) in the HPF and HTF sequences are indicated by an asterisk. Open circles indicate a deletion introduced for maximal alignment.

codons and a consensus splice donor sequence G↓GTGTGG and acceptor sequence CCTGTTATTTCAG↓T at each extremity separates the KRAB domain from the rest of the coding region (data not shown). This raises the possibility that elements A and B of the KRAB domain may correspond to separate exons.

KRAB shows enrichment in charged amino acids (i.e., 23 out of 75 amino acids are lysine, arginine, histidine, aspartic acid, or glutamic acid in HTF9) similar to that observed in the two homeobox-associated modules, the paired box and the POU domain (15–17), or the finger-associated boxes (18). We did not detect any other known DNA-binding motifs or protein–protein dimerization structures (2, 9–14); the heptad repeat of methionine/leucine residues reminiscent of a leucine-zipper structure that has been found in Kox1 ($MX_6LX_6LX_6L$, amino acid positions 43–67, where X is any amino acid; Fig. 2) (20) is not conserved in the other KRAB-finger sequences (29). However, by using the algorithm of Garnier *et al.* (30) and the method of Levin *et al.* (31), we detected two regions, corresponding to the more-conserved amino acid residues, that are able to fold into amphiphilic $\alpha$-helices with a pI value ranging from 8.6 in HPF4 to 3.8 in HTF12 (PC gene protein analysis package; Genofit, Geneva). As a number of observations have suggested that transactivating regions of several factors have an $\alpha$-helical structure (29), one attractive hypothesis is that the KRAB domain may function as a transcriptional activation domain.

This highly conserved KRAB domain is connected to the finger repeats by a less-conserved region of variable length; HTF and HPF cDNA clones are 76–97% similar in this region, whereas *Kox1*, *ZNF7*, *ZNF8*, *Zfp-1*, and *Xfin* are completely distinct. In most cases, this latter region is rich in cysteine, histidine, and tyrosine residues (14% in HTF9) and may thus correspond to degenerate finger repeats. All of our KRAB-finger proteins contain a large number of finger units (i.e., HPF4 contains 15 finger repeats), as do previously described KRAB-finger proteins (Xfin is the most remarkable example with 37 finger repeats). The finger repeats are located in the C-terminal regions (Fig. 2*A*). They all exhibit the characteristics of the Krüppel zinc-finger family and respect the strict consensus sequence T G E K P Y K/e C e E C G K A F n x S S x L T x H K x I H (where x is any amino

acid, capital letters correspond to amino acids conserved over 75%, and lowercase letters to amino acids conserved over 50%), although the first fingers of each protein deviate considerably from this consensus. By analogy to the C2/C2 steroid receptor proteins, these first fingers could be involved in determining target specificity (32).

As a first step to find out the functional role of these structurally related KRAB-finger genes, we have analyzed the expression pattern of individual mRNAs on Northern blots of total RNA from various human adult tissues or established cell lines as indicated (Fig. 3*A*). The complete cDNA insert used as a probe was first tested on a Southern blot of human genomic DNA cleaved by various restriction enzymes. By using highly stringent conditions (0.1× SSC/0.1% SDS at 65°C), each cDNA probe tested was found to hybridize with distinct genomic DNA fragments (data not shown). By using identical high-stringency conditions for Northern blot analysis, each probe tested hybridized with mRNAs about 4.4 kb long that were detectable in all samples analyzed. This size of 4.4 kb is similar to that described for the Kox1 (4.4 kb), Zfp-1 (4.8 and 2.2 kb), and Xfin (4.4 kb) KRAB-finger gene transcripts (20, 22, 23). Moreover, a 4.2-kb mRNA described as a cross-hybridizing transcript has also been detected by Kato *et al.* (28) for the human provirus-linked Krüppel gene (H-plk) closely related to the HPF9 cDNA clone. For each of the mRNAs, levels of expression were higher in the undifferentiated promyelocytic THP-1 and embryonic NT2D1 cell lines than in any normal tissue, with the lowest level observed in liver. Cross-hybridizing KRAB-bearing transcripts were also detected in the murine embryonal carcinoma cell line F9 (data not shown), suggesting that these genes, like *Zfp-1* in mouse, may be expressed early during embryonic development. As an internal control of the amount of RNA loaded in each lane, the filters were rehybridized with a human 28S rDNA probe (Fig. 3*B*) and with an oligo(dT) probe (data not shown).

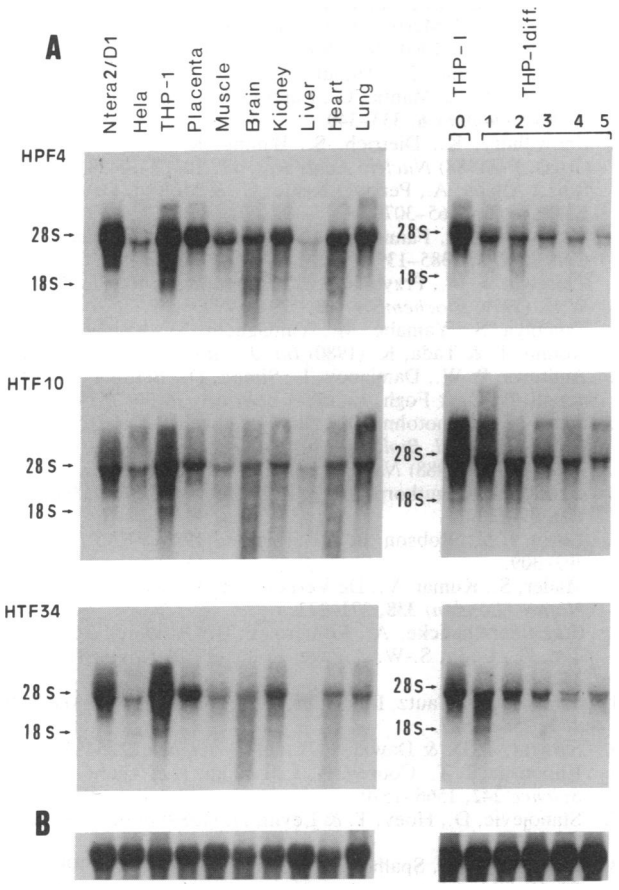


FIG. 3. Expression of KRAB-finger mRNAs HPF4, HTF10, and HTF34 (*A*) in various human tissues and cell lines as indicated and in THP-1 myeloid cells during *in vitro* THP-1 differentiation. Lanes: 0, undifferentiated cells; 1–5, PMA-treated cells and RNA extracted at 6 hr, 12 hr, 1 day, 3 days, and 5 days, respectively. The same blots were rehybridized with the 28S rDNA probe (*B*). The positions of rRNAs are indicated.

In overexposed autoradiographies, additional minor transcripts were also detected. These might correspond to related genes. Alternatively, as it has been reported for other finger genes (33, 34), they could represent alternate transcripts resulting from either the use of different promoters or from differential splicing. Comparison of HTF6 cDNA with an independently obtained 2A1.2 cDNA (R. Lovering and J. Trowsdale, personal communication) supports this hypothesis: the putative proteins differ only by the length of their KRAB domains (2A1.2 has the A and the B elements whereas HTF6 has only the B element).

Next, as the expression of potential developmental regulatory genes should be differentially regulated at critical events in development (35), we sought to determine whether the expression of the KRAB-finger genes was modulated during the process of *in vitro* differentiation. The human acute leukemia THP-1 cell line with phenotypic features of immature myeloid cells (26) was chosen as a model. Upon treatment with PMA, THP-1 cells cease to proliferate and differentiate along the macrophage lineage. Fig. 3*A* shows a Northern blot analysis of total RNA from untreated and PMA-treated THP-1 cells hybridized with individual KRAB-finger cDNAs. For each of the mRNAs analyzed, the relative amount of transcripts decreases 2- to 4-fold after treatment. To check for potential variation in the level of RNA electrophoresed, the blots were rehybridized with a human rDNA 28S probe (Fig. 3*B*) and with an oligo(dT) probe (data not shown). As comparable amounts were loaded, the down-regulation of the KRAB-finger genes observed after PMA stimulation of THP-1 cells appears to be significant. Consistent with our observation, a similar reduction of expression during *in vitro* differentiation of HL-60 cells has been reported for *ZNF7* and *ZNF8* KRAB-finger genes (21).

Since KRAB-related genes exist in mouse and *Xenopus*, we searched for KRAB-homologous sequences in other species. Southern blots of *Eco*RI-digested genomic DNA from various species, ranging from bacteria to humans, were hybridized with the HPF4 KRAB probe. By using the same low-stringency conditions that were used (no false positive found in six out of six positive clones analyzed) for library screening, multiple homologous DNA fragments were detected in all species tested except bacteria (Fig. 4). This suggests that several copies of KRAB-related sequences are conserved in the genome of all eukaryotes ranging from yeast to man. Moreover, as both KRAB and finger probes derived from HPF4 cDNA reveal many restriction fragments under identical hybridizing and washing conditions (Fig. 4), an unexpectedly large number of KRAB-finger genes may be present in all eukaryotes.

In conclusion, our results indicate that a set of ubiquitously expressed and differentially regulated genes coding for proteins containing a highly conserved KRAB domain connected to variable nucleic acid binding units is present in various eukaryotic organisms. By analogy to Krüppel and to other known nuclear regulatory finger proteins (3, 36, 37), the KRAB-finger genes may encode putative regulatory proteins involved in transcriptional regulation. One possible model is that the 75-amino acid N-terminal KRAB domain would provide a site for interaction with components of the transcriptional machinery resulting in activation or repression of gene expression. From recent evidence (i.e., HTF6 versus 2A1.2 cDNA; R. Lovering, personal communication), we

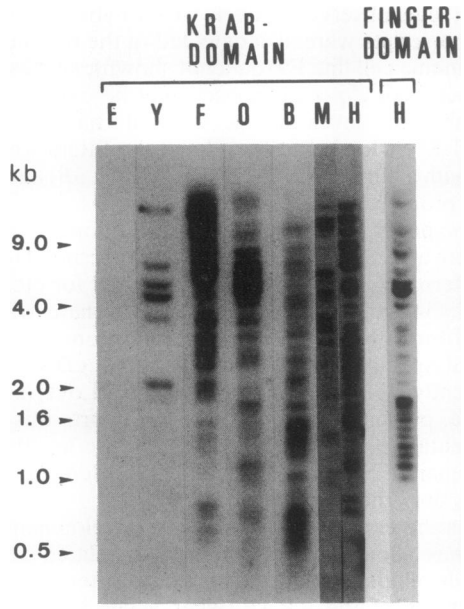


FIG. 4. Conservation of KRAB sequences across species. Southern blot of *Eco*RI-digested human (lane H), monkey (lane M), bovine (lane B), mouse (lane O), fish (lane F), yeast (lane Y), and bacterial (lane E) genomic DNA was hybridized with the KRAB or the finger probe from HPF4 cDNA under low-stringency conditions.

could further propose that differential promoter utilization or alternative splicing could lead to proteins containing the same zinc-finger domain that are either coupled or not coupled to the KRAB domain. The antagonistic activity that these proteins would likely display would add a further level of sophistication to the way they regulate gene expression. Such a situation has been reported for the human homeobox *HOX3C* gene† or the bovine papilloma virus *E2* gene (38).

†Mavilio, F., Arcioni, L., Simeone, A., Acampora, D. & Boncinelli, E., 16th EMBO Annual Symposium "The Molecular Biology of Vertebrate Development," Heidelberg, September 1990, p. 63 (abstr.).

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